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COMPLETE SPECIFICATION

Process for the Manufacture of Substances Preventing the Coagulation of Blood

We, ROCHE PRODUCTS LIMITED, a British Company, of Broadwater Road, Welwyn Garden City, Hertfordshire, do hereby declare that we are assignees of F. HOFFMAN-LA ROCHE & Co. AKTIENGESELLSCHAFT, a Swiss Company, of 124—184, Grenzacherstrasse, Basle, Switzerland, and that the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a process for the manufacture of substances which prevent the coagulation of blood hereinafter referred to as anticoagulants. The invention also includes novel anticoagulants.

Heparin, a polysaccharide sulphuric acid ester, may be obtained from livers and lungs and several processes are known for its manufacture from these materials. Most of these processes are alike insofar as there is first obtained a crude product, the so-called crude-heparin, from which a pure product, the so-called pure-heparin, can then be isolated or purified by various methods. For example, M. H. Kuizenga and L. B. Spaulding [J. Biol. Chem., 1943, 148, 641] obtained crude-heparin by autolysing cow lungs in the presence of ammonium sulphate, extracting the autolysate with sodium hydroxide, precipitating a heparin-protein complex by acidification of the extract with sulphuric acid, treating the heparin-protein complex with sodium hydroxide solution and, finally, precipitating the crude heparin from the solution so obtained by means of acetone. According to this known method 50 kg. of fresh lungs yields 102g. crude-heparin having an anticoagulating activity of 8.3 International Units per milligram (hereinafter denoted by the symbols: I.U./mg.). As to the isolation of pure-heparin the crude-heparin may be precipitated as its benzidine salt [F. Charles & A. R. Todd, Biochem. J., 1940, 34, 112.] or as its barium salt in dilute acetic acid [M. H. Kuizenga & L. B. Spaulding, loc. cit.]. In the method of isolation employing the barium salt technique, 102g. of crude-heparin containing 8.3 I.U./mg. gave 8.83g. of a barium compound having an activity of 85 I.U./mg. which corresponds to a yield of 88%, i.e. 15,000 I.U. per kg. lung. Therefore 12% of the activity was lost during the isolation. When further purifying this product by fractional precipitation these workers obtained a yield of 65% of pure-heparin having an activity of 125 I.U./mg. which corresponds to 9,750 I.U. per kg. lung. The whole procedure led to a loss of 40% of the heparin activity originally present.

According to J. E. Jorpes and S. Gardnell [J. Biol. Chem., 1948, 176, 267] the heparin contained in lungs and livers consists of sulphuric acid esters of polysaccharides of various degrees of esterfication—i.e. it consists of the so-called heparin-mono-, -di-, -tri- and, conceivably, the -tetra-sulphuric acid esters. The more sulphuric acid ester groups contained in the molecule, the higher is the anticoagulating activity. Heparin-monosulphuric acid ester, for instance, shows an activity of only 10—20 I.U./mg. During the purification process mentioned hereinbefore compounds of low activity, that is the lower so-called sulphuric esters of heparin, are lost and the reason for the fall of activity is due to this fact.

When crude-heparin is treated with zinc or cadmium chloride in dilute alcohol a product of low activity is obtained. Again, when the mother liquor obtained during the purification of crude-heparin as the benzidine salt is treated with four parts by volume of acetone a precipitate is formed which, on removal of the benzidine, gives a product

having 0.5 to 1.0 I.U./mg. Both these substances differ from the highly active heparin mainly in having a comparatively low sulphur content.

The manufacture of anticoagulants by the sulphation of certain natural or synthetic high polymers is known. Thus, the anticoagulant activity of synthetic polysaccharide sulphuric acid esters was shown by Bergstrom in 1936 [see Zeit. Physiol. Chemie, 1936, 238, 163]. By the sulphation of cellulose, starch, pectin and other compounds of like nature with chlorosulphonic acid he obtained preparations having an activity of the order of a fifth to a twentieth of that of heparin. Chargaff [see J. Biol. Chem., 1936, 115, 155] found that the activity of cellulose sulphuric acid ester and polyvinyl alcohol sulphuric acid ester was only about a fourth of that of heparin. P. Karrer [see Helv. Chim. Acta, 1943, 26, 1296] prepared substances having sulphur contents of 18—20% and an activity of 25 I.U./mg. and, starting from chondroitin sulphuric acid, he obtained substances having an activity of 17 I.U./mg. The activities attained by these workers were substantially lower than that of heparin which has an activity of 130 I.U./mg. Astrup [see Acta Physiol. Scand., 1944, 8, 215] concludes that cellulose tri-sulphuric acid ester and chitin-di-sulphonic acid are much less active than heparin. These hitherto known anticoagulants are of little value owing to their toxic side effects.

It has now been found, according to the present invention, that anticoagulants of an activity approximating that of pure-heparin [that is, the so-called heparin-tri- or tetra-sulphuric acid esters] can be obtained by sulphating lower heparin-sulphuric acid esters.

Accordingly a process is provided for the manufacture of blood anticoagulants which comprises sulphating a by-product of heparin manufacture, that is, a so-called sulphuric acid ester having a sulphur content less than that of heparin tri-sulphuric acid ester.

As starting materials in the present process the by-products of heparin manufacture having comparatively low activity may be used. Thus, for example, any of the following are suitable for this purpose:—

(1) The by-product which is separable from impure heparin preparations by means of treatment with zinc and cadmium salts in dilute alcohol. [Starting material A].

(2) Heparin-mono- or -di-sulphuric acid ester. [Starting Material B.]

(3) The total fraction which is precipitated by alcohol or acetone during heparin manufacture. [Starting material C.]

(4) The fraction which is obtainable from the mother liquors of the heparin purification process. [Starting Material] D].

As sulphating agent chlorosulphonic acid can be used. The sulphation may be carried out in a solvent such as, for example, pyridine or α-picoline.

The process may be carried out using the by-products of heparin manufacture singly or in admixture and the process may also be carried out in the presence of heparin.

The yield of substances of high activity obtained by the present process is higher than any hitherto obtained—thus 50,000 I.U. and higher amounts can be obtained from 1 kg. of lungs.

The removal of excess sulphating agent from the reaction mixture during the isolation of the sulphated products of the invention presents some difficulty. According to P. Karrer [see Helv. Chim. Acta, 1943, 26, 1296] removal of sulphuric acid may be achieved by dialysis. ~~It has been found preferable~~ however to simply precipitate the sulphated products from the reaction mixture by means of alkaloids such as, for example, brucine or narcotine.

Apart from the process hereinbefore described the present invention comprises novel substances which differ from heparin. These substances have an activity of more than 100 I.U./mg. and contain from 10—15% sulphur. One such substance is a laevo-rotatory product obtained by sulphation of the fraction separable from crude-heparin by treatment with zinc or cadmium salts. This substance shows an $[\alpha]^{20}_{D}$ ranging from about $-10°$ to about $-30°$. Another such substance is a dextro-rotary product having an $[\alpha]^{20}_{D}$ of less than 40° obtained by sulphation of the total fraction precipitated from crude-heparin with alcohol or acetone. Yet another such substance is an almost optically inactive product obtained by the sulphation of that fraction which can be precipitated from the mother liquors of heparin manufacture by means of organic solvents, such as alcohol or acetone.

The following examples, in which the parts by weight and parts by volume are in corresponding e.g.s. units, are illustrative of the way in which the present invention can be put into effect:—

EXAMPLE 1

1 Part by weight of starting material

1. New blood anticoagulants having an activity of more than 100 International Units per milligram and a sulphur content of from 10% to 15%, which comprise sulphated by-products of heparin manufacture.

2. A new blood anticoagulant having the characteristics set forth in claim 1 and which is laevo-rotatory which comprises a sulphated fraction obtainable from a solution of crude-heparin in alcohol by treatment with zinc and cadmium salts.

3. A new blood anticoagulant having the characteristics set forth in claim 1, which comprises a further sulphated heparin-mono- or heparin-di-sulphuric acid ester.

4. A new blood anticoagulant having the characteristics set forth in claim 1 and which has a rotation of less than $[\alpha]^{D}_{20} = +40°$, which comprises a sulphated total fraction obtainable from crude-heparin by precipitation with alcohols or acetone.

5. A new blood anticoagulant having the characteristics set forth in claim 1 and which shows little or no optical activity, which comprises a sulphated fraction precipitated from the mother liquors of heparin manufacture by addition of alcohols or acetone.

6. A process for the enhancement of the blood anticoagulant activity of heparin preparations which contain substances of lower activity than that of heparin itself which comprises treating said preparations with a sulphating agent.

7. A process for the manufacture of blood anticoagulants which comprises treating a by-product of heparin manufacture having an activity of the order of that of heparin-mono- or -di-sulphuric acid ester with a sulphating agent to produce products having an activity of more than 100 International Units per milligram and a sulphur content of from 10% to 15%.

8. A process for the enhancement of the blood anticoagulant activity of by-products of heparin manufacture which comprises treating said by-products with a sulphating agent.

9. A process in accordance with claim 7 or claim 8 wherein the said by-product is that obtained by treating crude-heparin in dilute alcohol with zinc or cadmium salts.

10. A process in accordance with claim 7 or claim 8 wherein the said by-product is heparin-mono- or heparin-di-sulphuric acid ester or a mixture thereof.

11. A process in accordance with claim 7 or claim 8 wherein the said by-product is that obtained from crude-heparin by precipitation with alcohols or acetone.

12. A process in accordance with claim 7 or claim 8 in which the said by-product is that obtained from the mother liquors of heparin manufacture by precipitation with alcohols or acetone.

13. A process in accordance with any one of claims 6 to 12 inclusive wherein the sulphating agent used is chlorosulphonic acid.

14. A process in accordance with any one of claims 6 to 13 inclusive which includes the additional step wherein the product or products of sulphation are isolated by precipitation with an alkaloid.

15. A process in accordance with claim 14 wherein the alkaloid is brucine or narcotine.

16. A process for the enhancement of the blood anticoagulant activity of heparin preparations and of by-products of heparin manufacture substantially as described.

17. A process for the manufacture of blood anticoagulants substantially as described with reference to examples 1 to 4 herein.

For ROCHE PRODUCTS LIMITED:

W. D. Whitaker.

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LE 1
f starting material

A with an activity of 50 I.U./mg. which may be obtained according to the method mentioned above, is stirred with a solution of 1 part by volume of chlorosulphonic acid in 10 parts by volume of pyridine which has been warmed to 80° C. The mixture is left standing for 10 minutes and the supernatant solution is then removed. The residue is dissolved in 100 parts of water and the aqueous solution is brought to pH$=3$ by the addition of hydrochloric acid. 50 parts by volume of a 10% aqueous solution of narcotine hydrochloride are added with stirring, thereby precipitating the narcotine salt of the sulphuric acid ester in form of coarse flakes. The mixture is centrifuged and the residue washed 3 times with 10 parts by volume of a 1% aqueous solution of narcotine hydrochloride. The narcotine salt is suspended in 10 parts of water and it is added with 10 parts by volume of a 10% sodium carbonate solution. The narcotine base which separated is filtered, the filtrate is brought to pH$=6$ by means of acetic acid and the sulphuric acid ester is precipitated by means of twice the volume of methanol. After drying with alcohol and ether there are obtained 1.1 parts by weight of a product which contains 14.5% sulphur and which shows a titer of 130 I.U./mg., thus corresponding to the international heparin standard. Optical activity $[\alpha]^{20}_{D} = -17°$.

EXAMPLE 2

1 Part by weight of starting material B of a titer of 25 I.U./mg., which may be obtained according to the method mentioned above, is introduced portionwise into a solution of 2 parts by volume of chlorosulphonic acid in 10 parts by volume of α-picoline which has been warmed to 70° C. The mixture is stirred 4 hours at 70° C. and then it is added with 100 parts of water. The clear solution is adjusted to pH$=3$ by means of 4 parts by volume of concentrated hydrochloric acid and it is then added with 80 parts by volume of a 10% aqueous solution of brucine hydrochloride. The brucine salt of the new sulphuric acid ester is centrifuged off and washed 3 times with 10 parts by volume of a 1% aqueous solution of brucine hydrochloride. The brucine salt is decomposed by 20 parts by volume of 5% sodium hydroxide and the brucine base precipitated is separated by filtration. The filtrate is brought to pH$=5$ by means of acetic acid and the sulphuric acid ester is precipitated with twice the volume of ethanol. Upon drying with alcohol and ether there are obtained 1.2 parts by weight of a product showing a titer of 120 I.U./mg.

EXAMPLE 3

1 Part by weight of starting material C with a titer of 10 I.U./mg., as it may be obtained according to the method mentioned above in form of the raw heparin, is introduced portion by portion into a solution of 2 parts by volume of chlorosulphonic acid in 10 parts by volume of pyridine which has been warmed up to 100° C. After 15 minutes standing 100 parts of water are added. The solution is brought to pH$=3$ by means of concentrated hydrochloric acid and 80 parts by volume of a 10% aqueous solution of narcotine hydrochloride are added. The mixture is centrifuged and the narcotine salt is washed 3 times with 10 parts by volume of a 1% aqueous solution of narcotine hydrochloride. The residue is decomposed by means of 20 parts by volume of a 5% sodium carbonate solution. The narcotine precipitated is separated by filtration. The filtrate is adjusted to pH$=5$ by means of acetic acid and the sulphuric acid ester is precipitated with twice the volume of methanol. Upon drying with alcohol and ether there is obtained 1 part by weight of a preparation of a titer of 110 I.U./mg. and a sulphur content of 14.2%. Optical activity $[\alpha]^{20}_{D} = +25°$.

EXAMPLE 4

1 Part by weight of starting material D of a titer of 0.5 to 1 I.U./mg., as obtained according to the procedure mentioned above, is stirred for 5 hours at 60° C. with 2 parts by volume of chlorosulphonic acid in 10 parts by volume of pyridine 200 parts of water are added and the pH is adjusted to 3.5 with concentrated hydrochloric acid. The sulphonation product is precipitated by the addition of 80 parts by volume of a 10% aqueous solution of narcotine hydrochloride. The precipitate is sucked off and washed 3 times with 10 parts by volume of a 1% aqueous solution of narcotine hydrochloride. The narcotine salt is treated with 20 parts by volume of a 5% aqueous sodium carbonate solution and the narcotine base which precipitates is separated. The sulphonation product is isolated from the filtrate which is adusted to pH 5 by means of 2 parts by volume of alcohol. Upon drying with alcohol and ether there are obtained 1.1 parts by weight of a compound with a titer of 135 I.U./mg. and a sulphur content of 13.1%. Optical activity $[\alpha]^{20}_{D} = -3°$.

What we claim is:—